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Obesity is linked to chronic inflammation in white adipose tissue (WAT) which is exacerbated by infiltrating macrophages (MΦ)s. Grape powder extract (GPE) contains polyphenols that have the potential to prevent this inflammatory response that can otherwise lead to insulin resistance. Therefore, this research examined the extent to which GPE prevents lipopolysaccharide (LPS)-induced inflammatory response in human MΦs. Pretreatment of human MΦs with GPE prevented LPS-mediated activation of mitogen activated protein kinases (MAPK)s, nuclear factor kappa B (NFκB), and activator protein (AP)-1, and induction of inflammatory genes. Furthermore, GPE decreased the capacity of LPS-stimulated MΦs to inflame adipocytes and cause insulin resistance. My preliminary data show that in the absence of LPS, quercetin (QUE), a polyphenol abundantly found in GPE, attenuated inflammatory gene expression, c-Jun N-terminal kinase (JNK) and c-Jun activation, and IκBα degradation in human MΦs. In addition, QUE pretreatment of MΦs prevented insulin resistance in human adipocytes exposed to MΦ-CM. Taken together, these data show that polyphenol-rich GPE decreases inflammation in human MΦs and MΦ-mediated insulin resistance in human adipocytes. Lastly, these data suggest that QUE may be one of the polyphenols in GPE that decrease inflammation and insulin resistance in human MΦs and adipocytes.

GRAPE POWDER EXTRACT (GPE) ATTENUATES
MARKERS OF INFLAMMATION IN
HUMAN MACROPHAGES

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Overview

This thesis is part of an ongoing project whose *long term goal* is to identify potential health benefits of a polyphenol-rich grape powder extract (GPE). These studies are expected to identify dietary strategies for controlling chronic inflammation and insulin resistance associated with obesity. The incidence of obesity is increasing worldwide and due to its associated comorbidities, is becoming a major public health concern (reviewed in Tilg et al. 2006 and Poirier et al. 2006). Obesity is associated with a low-grade, chronic inflammatory disease characterized by macrophage (MΦ) infiltration into white adipose tissue (WAT) (reviewed in Cottam et al. 2004 and Trayhurn et al. 2004). Secretion of proinflammatory mediators from activated MΦs exacerbates WAT inflammation, thereby contributing to the pathogenesis of obesity-related diseases such as atherosclerosis, type 2 diabetes, and hypertension (Laine et al. 2007) (**Fig. 1.1**). Therefore, preventing MΦ inflammation with bioactive components in grapes could help prevent or attenuate the severity of these obesity-related diseases.

Grapes are one of the most widely consumed fruits in the world (Sakurai et al. 2009). They contain a variety of polyphenols such as quercetin (QUE) that have anti-oxidant and anti-inflammatory properties. For example, QUE (Stewart et al. 2008, Rivera et al. 2008) has been shown to reduce inflammation and insulin resistance in

rodent models. However, the specific bioactive component(s) in GPE and the mechanism(s) by which they prevent inflammation or improve insulin sensitivity are still unclear.

Central Hypothesis and Specific Aim

The *central hypothesis* tested in this thesis was that GPE will prevent inflammation in human MΦs by blocking the activation of mitogen activated protein kinases (MAPK)s, activator protein (AP)-1, and nuclear factor kappa B (NFκB) (**Fig 1.2**). Cross-talk between MΦs and adipocytes was also examined, because MΦs have been reported to cause inflammation and insulin resistance in adipocytes. The specific aim of this study was to *determine the extent to which GPE prevents inflammation in human MΦs*. The *working hypothesis* for this aim was that GPE will block lipopolysaccharide (LPS)-mediated activation of MAPKs [i.e., c-Jun N-terminal kinase (JNK), p38], NFκB, or AP-1 that increase the transcription of inflammatory genes in human MΦs. In addition, GPE will decrease the capacity of LPS-stimulated MΦs to inflame adipocytes and cause insulin resistance.

The *expected outcomes* of this specific aim were that GPE will decrease markers of inflammation (i.e., gene expression, protein secretion) in MΦs by blocking LPS-mediated activation of JNK, p38, NFκB, or AP-1 (i.e. c-Jun). Furthermore, GPE will decrease the capacity of LPS-stimulated MΦs to inflame adipocytes and cause insulin resistance. Collectively, these outcomes are expected to promote the development of novel dietary strategies to prevent or attenuate obesity-associated inflammation.

Background and Significance

Obesity-related inflammation and chronic disease are the most prevalent nutrition-related health problems in the U.S. (Anon 2007). Obesity-associated inflammation is characterized by an increased abundance of MΦs in WAT, leading to the production of inflammatory cytokines, chemokines, and prostaglandin (PG)s that can cause insulin resistance. *What is needed is a naturally-occurring food or dietary component without side effects that prevents chronic inflammation or insulin resistance.* Grapes are one of the most widely consumed fruits in the world and they contain high concentrations of polyphenols which have been shown *in vivo* and *in vitro* to exert anti-inflammatory and anti-oxidant actions (Sakurai et al. 2009). *However, the ability of GPE and its polyphenols to prevent inflammation in human MΦs and block inflammation or cross-talk with human adipocytes and their mechanism of action are unknown.* As an outcome of the proposed investigations, I expect to have determined a mechanism by which GPE attenuates inflammation in human MΦs. This will result in decreased inflammation and insulin resistance in adipocytes. *The research proposed is significant, because it could potentially lead to improvements in the nation's health and nutrition and reduce health care costs.*

Review of Literature

Metabolic endotoxemia (i.e., high plasma levels of LPS) is associated with obesity (Cani et al. 2008), which contributes to inflammation and insulin resistance. LPS activates MAPKs, NFκB, and AP-1, increasing the release of inflammatory cytokines and chemokines (Terra et al. 2007, Jang et al. 2008, Chen et al. 2007). In obesity, the increased production of these inflammatory mediators originates mainly from infiltrating MΦs in WAT (Xu et al. 2003, Weisberg et al. 2003). MΦ-secreted factors have been shown to increase inflammation and decrease insulin-stimulated glucose uptake in adipocytes (Lumeng et al. 2007, Permana et al. 2006). Consistent with these findings, my data demonstrated that LPS increased the activation of MAPK, NFκB, and AP-1 in human MΦs, increasing their capacity to cause inflammation and insulin resistance in primary human adipocytes.

Recent attention has focused on the anti-inflammatory properties of bioactive food components. Because fruits and fruit extracts are abundant in bioactive components such as polyphenols, they possess an immense potential to prevent the development of obesity-related inflammation and insulin-resistance. Several grape products have been shown to reduce markers of inflammation or oxidative stress associated with cardiovascular disease *in vivo*, *ex vivo*, and *in vitro* (reviewed in Perez-Jimenez et al. 2008 and Leifert et al. 2008). For example, consumption of red grape juice by hemodialysis patients, who suffer from hemodialysis-induced oxidative stress, reduced plasma levels of oxidized LDL and *ex vivo* neutrophil NADPH oxidase activity (Castilla et al. 2008). Similarly, consumption

of red grape juice decreased markers of oxidative stress in healthy adults (O'Bryne et al. 2002). Consumption of grape powder by adult women decreased the levels of plasma tumor necrosis factor (TNF)- α and urinary F2-isoprostanes, markers of inflammation and oxidative stress, respectively (Zern et al. 2005). Similarly, adults consuming red wine had lower urinary levels of PGF- $_{2\alpha}$ levels and higher plasma levels of polyphenols compared to those consuming white wine or no wine (controls) (Pignatelli et al. 2006).

Salt-sensitive, hypertensive Dahl rats supplemented with grape powder had decreased cardiac oxidative damage and plasma levels of interleukin (IL)-6 and TNF α compared to unsupplemented control rats (Seymour et al. 2008). Zucker rats fed a high fat diet supplemented with grape seed procyanidins had lower plasma levels of C-reactive protein (CRP) and lower WAT mRNA levels of CRP, TNF α , and IL-6 compared to high fat fed control rats (Terra et al. 2009). Apolipoprotein E deficient mice, who suffer from atherosclerosis and oxidative stress, supplemented with grape powder had a lower incidence of atherosclerotic plaque formation and decreased markers of oxidative stress compared to controls (Fuhrman et al. 2005). Furthermore, consumption of grape powder by apolipoprotein E deficient mice decreased the capacity of their peritoneal M Φ s to oxidize LDL. Consistent with these data, grape seed proanthocyanidin supplementation to cholesterol-fed hamster's decreased M Φ -mediated, aortic foam cell development by 50% compared to the unsupplemented controls (Vinson et al. 2002).

In vitro, grape seed procyanidins decreased cholesterol and TG accumulation in MΦ-derived foam cells (Terra et al. 2009) and modulated the inflammatory response in endotoxin-stimulated RAW264 MΦs by inhibiting NFκB (Terra et al. 2007). In addition, human MΦ (THP-1 cell line) and adipocytes (SGBS cell line) treated with grape seed procyanidins had reduced expression of IL-1 and monocyte chemoattractant protein (MCP)-1 and decreased activation of NFκB following stimulation with LPS or TNFα, respectively (Chacón et al. 2009). Moreover, oligomerized grape seed polyphenols have been shown to reduce NFκB transcriptional activity and activation of extracellular signal-related kinase (ERK) in a co-culture of adipocytes and MΦs (Sakurai et al. 2009). Consistent with these findings, my preliminary data show that GPE attenuates LPS-mediated inflammatory gene expression in MΦs, possibly by decreasing activation of JNK, p38, c-Jun, Elk-1, or NFκB.

However, the ability of GPE to prevent inflammation in human MΦs and block inflammation and insulin resistance in human adipocytes treated with conditioned media (CM) from activated MΦs and its mechanism of action are unknown. Thus, experiments described in the next chapter were designed to test the *central hypothesis* and carry out the *specific aim of this project*.

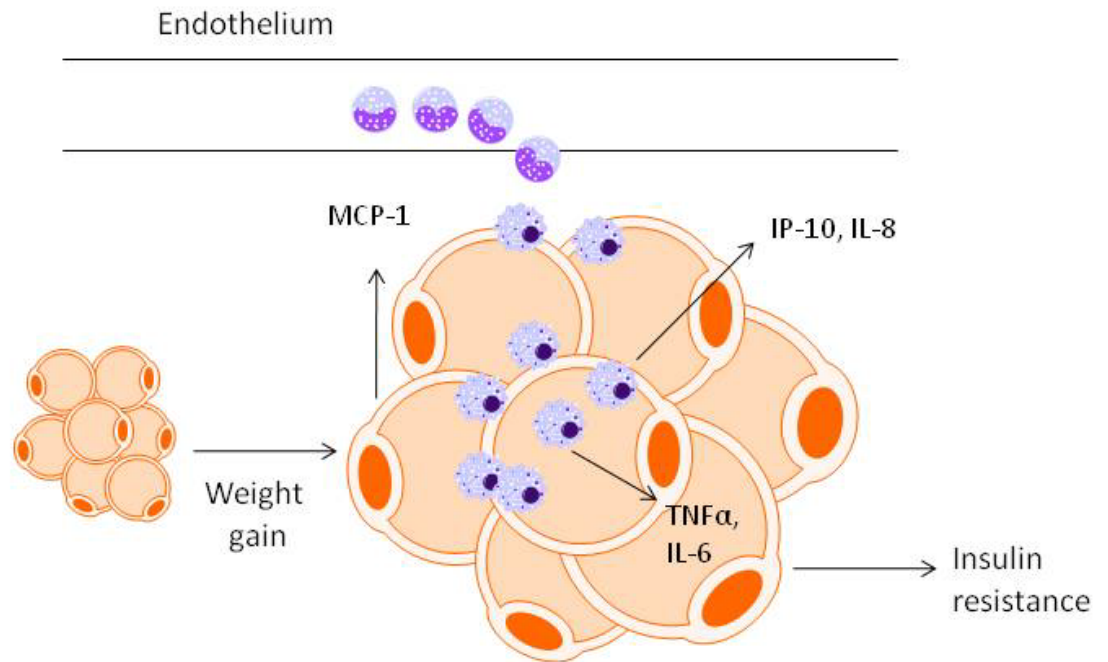


Fig 1.1. MΦ recruitment into WAT. Weight gain results in hypertrophic adipocytes that release chemokines such as monocyte chemoattractant protein (MCP-1) that recruit MΦs into WAT. Activated infiltrated MΦs, in turn, release chemokines (IP-10, IL-8) and cytokines (TNFα, IL-6) amplifying the inflammatory response in WAT, leading to insulin resistance.

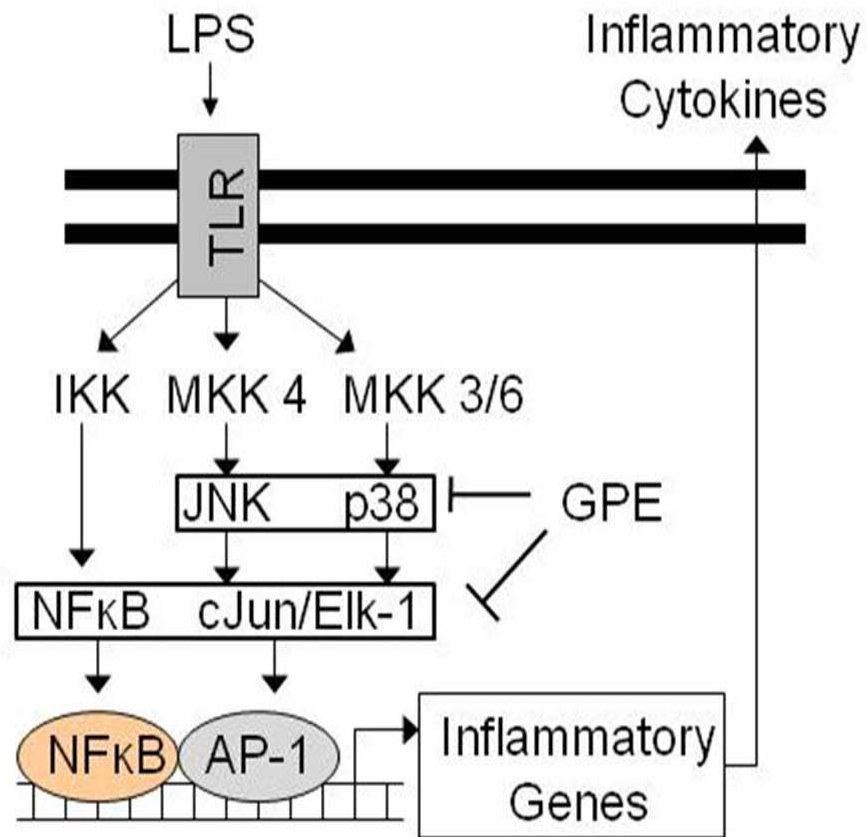


Fig 1.2. Proposed mechanism by which GPE prevents inflammatory gene expression in human MΦs. GPE blocks LPS-mediated activation of c-Jun N-terminal kinase (JNK), p38, NFκB, or activator protein AP-1 (i.e. c-Jun).

CHAPTER II

POLYPHENOL-RICH GPE ATTENUATES INFLAMMATION IN HUMAN MACROPHAGES (MΦ)S AND IN HUMAN ADIPOCYTES EXPOSED TO MΦ-CONDITIONED MEDIA

Abstract

Obesity-associated inflammation is characterized by an increased abundance of MΦs in WAT, leading to the production of inflammatory cytokines, chemokines, and PGs that can cause insulin resistance. GPE is rich in phenolic phytochemicals that possess anti-oxidant and anti-inflammatory properties. Thus, I examined the ability of GPE to prevent LPS-mediated activation of MAPKs, NFκB, and AP-1, and induction of inflammatory genes in human MΦs. In addition, I investigated the extent to which GPE pretreatment of MΦs prevented inflammation and insulin resistance in human adipocytes incubated with MΦ-conditioned media (MΦ-CM). Pretreatment of MΦs with GPE attenuated LPS-induction of inflammatory cytokines such as TNFα, interleukin IL-6, and IL-1β, chemokines such as IL-8 and interferon (IFN)γ inducible protein (IP)-10, and a marker of PG production, cyclooxygenase (COX)-2. GPE also attenuated LPS activation of MAPKs, NFκB, and AP-1 (c-Jun) as evidenced by decreased 1) phosphorylation of JNK and p38, 2) degradation of IκBα and activation of an NFκB reporter construct, and 3) phosphorylation of c-Jun and Elk-1. Using LPS-challenged MΦ-CM, GPE pretreatment attenuated MΦ-mediated inflammatory gene expression, activation of an

NFκB reporter, and suppression of insulin-stimulated glucose uptake in human adipocytes. Collectively, these data demonstrate that GPE attenuates LPS-mediated inflammation in human MΦs, possibly by decreasing the activation of MAPK, NFκB, and AP-1, and that GPE decreases the capacity of LPS-stimulated MΦs to inflame adipocytes and cause insulin resistance.

Introduction

The incidence of obesity is increasing worldwide and due to its associated comorbidities, is becoming a major public health concern (reviewed in Tilg et al 2006 and Poirier et al. 2006). Obesity is considered a low-grade, chronic inflammatory disease characterized by macrophage (MΦ) infiltration to WAT (reviewed in Cottam et al. 2004 and Trayhurn et al. 2004). Secretion of proinflammatory mediators from activated MΦs exacerbates WAT inflammation, thereby contributing to the pathogenesis of obesity-related diseases such as atherosclerosis, type 2 diabetes, and hypertension (Laine et al. 2007). Indeed, obese individuals present with increased serum and tissue levels of inflammatory markers such as TNFα, IL-6, and MCP-1 (Wellen et al. 2005, Fantuzzi et al. 2004). Notably, several studies reported that MΦs are the primary source for proinflammatory cytokine production in WAT and may be recruited to WAT via MCP-1 (Fain et al. 2004, Clément et al. 2004, Curat et al. 2006, Kanda et al. 2006, Skurk et al. 2005). Furthermore, numerous animal studies have demonstrated the importance of MΦs in inflammation-induced insulin resistance (Kanda et al. 2004, Lumeng et al. 2007, Arkan et al. 2005, Weisberg et al. 2005).

High blood levels of LPS are indicative of metabolic endotoxemia (Cani et al. 2008), and activate immune cells, including MΦs. LPS activates toll-like receptors (TLR) and co-receptors (CD14, MD-2) on these cells, triggering an inflammatory signaling cascade involving MAPKs, NFκB, Elk-1, and AP-1. These activated proteins induce the transcription of many proinflammatory genes including cytokines, chemokines, and other immunomodulators that adversely affect WAT, triggering inflammation and insulin resistance. Notably, diet-induced obesity increases plasma levels of LPS in mice, which contributes to obesity, inflammation, and insulin resistance in these animals (Cani et al. 2008). Therefore, reducing chronic inflammatory cytokine production through inhibition of the MAPK and NFκB signaling pathways in MΦs may prevent obesity-associated inflammation and insulin resistance.

Recent attention has focused on the anti-inflammatory properties of bioactive food components. Because fruits and fruit extracts are abundant in bioactive components such as polyphenols, they possess an immense potential to prevent the development of obesity-related inflammation and insulin-resistance. Grapes are one of the most widely consumed fruits in the world (Sakurai et al. 2009). They contain high concentrations of polyphenols which have been shown in numerous *in vivo* and *in vitro* studies to exert anti-inflammatory and anti-oxidant effects. For example, grape seed procyanidin modulated the inflammatory response in endotoxin-stimulated RAW264 MΦs by inhibiting NFκB (Terra et al. 2007). In addition, oligomerized grape seed polyphenols have been shown to reduce NFκB transcriptional activity and activation of ERK in a co-culture of murine adipocytes and MΦs (Sakurai et al. 2009). However, the ability of GPE) to prevent

inflammation in human MΦs and block inflammation and insulin resistance in human adipocytes treated with MΦ-CM is unknown. Furthermore, the potential anti-inflammatory mechanisms of action of GPE are unknown.

In the present study, we hypothesized that GPE would prevent LPS-mediated activation of MAPK, NFκB, Elk-1 and AP-1, and subsequent induction of inflammatory genes in human MΦs. Furthermore, we speculated that GPE would decrease inflammation and insulin resistance in primary cultures of human adipocytes incubated with LPS-challenged MΦ-CM.

Materials and Methods

Materials

All cell culture-ware were purchased from Fisher Scientific (Norcross, GA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). RPMI 1640 was purchased from ATCC (Manassas, VA). LPS was purchased from Sigma-Aldrich (St. Louis, MO). Tri Reagent was purchased from Molecular Research Center (Cincinnati, OH). Gene-specific primers were purchased from Applied Biosystems (Foster City, CA). Polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho (Thr180/Tyr182) and total p38 antibodies were purchased from BD Bioscience Pharmingen (San Jose, CA). Anti-phospho (Thr183/Tyr185) and total c-jun NH₂-terminal kinase (JNK), anti-phospho (Ser63) c-Jun and total c-Jun, anti-phospho (Ser383) Elk-1, and anti- IκBα antibodies were purchased from Cell Signaling Technologies (Beverly,

MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA). All other reagents and chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Culturing of human MΦs

Human U937 monocytes were purchased from ATCC (Manassas, VA). Cells were seeded in 35-mm dishes at 0.75×10^6 cells per 35 mm or 1.25×10^6 cells per 60 mm dish and differentiated with 30 $\mu\text{g/L}$ phorbol 12-myristate (PMA) for 24 h in RPMI 1640 (containing 10% FBS, 60 U/mL^{-1} penicillin, 60 U/L^{-1} streptomycin and 25 $\mu\text{g/mL}^{-1}$ amphotericin B). Media was then changed to PMA-free RPMI and 24 h later the experiments were initiated with the MΦ monolayers. Cultures were incubated at 37°C in a humidified $\text{O}_2\text{:CO}_2$ (95:5%) atmosphere.

Culturing of human primary adipocytes

Abdominal WAT was obtained from non-diabetic Caucasian and African American females, between the ages of 20-50 years old with a body mass index (BMI) less than 32.0 $\text{kg (m}^2\text{)}^{-1}$ following abdominoplasty. Approval was obtained from the Institutional Review Board at the University of North Carolina at Greensboro and the Moses Cone Memorial Hospital in Greensboro, NC. Tissue was digested using collagenase and stromal vascular (SV) cells were isolated and cultured as previously described (Brown et al. 2004). MΦ-free cultures containing ~50% preadipocytes and

~50% adipocytes, based on visual observations and previous analyses (Chung et al. 2006), were treated between day 6-12 of differentiation. Each experiment was repeated at least twice at different times using a mixture of cells from 2-3 subjects unless otherwise indicated.

Source and preparation of GPE

Lyophilized grape powder, obtained from red, green, and blue-purple seeded and seedless California (CA) table grapes, was acquired from the California Table Grape Commission (CTGC). This grape powder has been reported to contain several types of polyphenols including anthocyanins, monomeric flavanols, flavonols, and stilbenes (Seymour et al. 2008). The powder was extracted to remove the sugars (90% w/w) using a Diaion HP-20 anion resin column and eluted from the column using methanol, and subsequently lyophilized per a protocol provided by the CTGC. GPE was dissolved in dimethyl sulfoxide (DMSO) to make the concentration of 100 mgmL⁻¹ as the stock solution and stored at -20°C. Stock concentrations were diluted immediately prior to use.

Analysis of GPE polyphenols

Several polyphenols in GPE were determined (**Fig. 2.1**) using reverse phase high-performance liquid chromatography (HPLC) as previously described (Xie et al. 2007).

RNA isolation and real-time quantitative PCR (qPCR)

Following treatment, cells were harvested and total RNA was isolated using Tri-Reagent according to the manufacturer's protocol. For real time qPCR, 2.0 µg total RNA was converted into first strand cDNA using Applied Biosystems High-Capacity cDNA Archive Kit. The qPCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously quantified for each sample, and data were normalized accordingly.

Analysis of secretion of IL-6 and interferon- γ inducible protein-10 (IP-10) through multiplex cytokine assay

The concentrations of IL-6 and IP-10 were determined using the BioPlex® Suspension Array System from Bio-Rad (Hercules, CA) following the manufacturer's protocol.

SDS-page western immunoblotting analysis

After experimental treatments, the medium was removed quickly, and the monolayers were washed gently once with ice-cold HBSS. RIPA lysis buffer was then added containing Tris HCL (pH 7.5), 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM EDTA with protease and phosphatase inhibitors. Monolayers were immediately scraped, transferred to prechilled microfuge tubes, and triturated to break up cells. Cell

lysates were then sonicated three times for 5 s. Cell debris was pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C, and the resulting supernatant was collected for analysis. The protein concentration of each sample was determined using a BCA assay. SDS-PAGE and transfer of protein to polyvinylidene difluoride membranes were carried out on the same day as the protein harvest to prevent freeze-thaw degradation of phosphoproteins. After transfer, membranes were blocked in Tris-buffered saline (TBS), supplemented with 0.1% Tween 20 (TBS-T) and 5% nonfat dried milk for 1 h at room temperature. The membranes were washed twice with TBS-T and incubated in TBS-T supplemented with 5% BSA and the following primary antibodies overnight at 4 °C: rabbit polyclonal Anti-phospho (Thr183/Tyr185) and total c-jun NH₂-terminal kinase (JNK), anti-phospho (Ser63) c-Jun and total c-Jun, anti-phospho (Ser383) Elk-1, and anti-IκBα antibodies all from Cell Signaling Technologies, mouse monoclonal anti-phospho (Thr180/Tyr182) and total p38 from BD Transduction Laboratories, and goat polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology . The next morning, primary antibody was washed three times for 5 min with TBS-T, and the membranes were immediately incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. After three washes with TBS-T, chemiluminescence was initiated by the addition of Western Lightning Plus (Perkin Elmer Life Sciences) and subsequent exposure to x-ray film.

Transient transfections of MΦs

Differentiated U937 cells were transiently transfected with the NFκB responsive luciferase (luc) reporter construct pNFκB luc (Stratagene, La Jolla, CA) using the Amaxa Nucleofector. 1.25×10^6 cells from a 60 mm plate were collected by scraping with 10 mmol/L EDTA and resuspended in 100 μL of nucleofector solution (Amaxa) and mixed with 2 μg of pNFκB luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the W-005 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 min recovery in RPMI media. Following 24 h, transfected cells were pretreated with $10 \mu\text{g mL}^{-1}$ GPE for 1 h and treated with 100 ng mL^{-1} LPS for 8 h. This 8 h treatment with LPS was chosen based on the results of a pilot time course study (data not shown). Firefly luciferase activity was measured using the Dual-Glo luciferase kit and normalized to *Renilla* luciferase activity from the cotransfected control pRL-CMV vector. All luciferase data are presented as a ratio of firefly luciferase to *Renilla* luciferase activity.

MΦ CM experiments in primary human adipocytes

CM was collected from differentiated U937 cultures pretreated for 1 h with or without 10, 30, 60, or $100 \mu\text{g mL}^{-1}$ GPE followed by treatment for 3 h with 100 ng mL^{-1} LPS. MΦ-CM obtained from each experiment was pooled and stored at -80°C until used. Distinct pools were used for each experiment. For RNA isolation and qPCR experiments, primary human adipocytes were seeded in 35-mm dishes at 0.5×10^6 per dish and allowed to differentiate for 6 d. On day 6, media was changed and cells were incubated in

1 mL of AM-1. Twenty-four hours later, the following were added to the cultures 1) fresh adipocyte medium (AM-1), 2) fresh RPMI, 3) LPS challenged MΦ-CM, or 4) LPS-challenged MΦ-CM pretreated with GPE. The amount and duration of MΦ-CM treatment varied depending on the outcome measured.

Transient transfections of primary human adipocytes

Primary human adipocytes were transiently transfected with the NFκB responsive luciferase (luc) reporter construct pNFκB luc (Stratagene) using the Amaxa Nucleofector. On day 6 of differentiation, 1.2×10^6 cells from a 60 mm plate were trypsinized and resuspended in 100 μL of nucleofector solution (Amaxa) and mixed with 1 μg of pNFκB luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 min recovery in calcium-free RPMI media. Following 24 h, transfected cells were treated for 24 h with GPE- or LPS- MΦ-CM as described above. This 24 h treatment with LPS- MΦ-CM was chosen based on the results of a pilot time course study (data not shown). Firefly and *Renilla* luciferase activity were measured as described above for the transfected MΦs.

2-[³H]deoxy-glucose (2-DOG) uptake

Primary human adipocytes were incubated with low glucose (5 mmol/L) and insulin (20 pmol/L)-containing media for 24 h. Cultures were then treated with LPS challenged MΦ-CM or CM from LPS-challenged MΦ pretreated with GPE for 24 h.

Basal and insulin-stimulated 2-DOG were measured as described previously (Brown et al. 2004).

Statistical analysis

Statistical analyses were performed for data in Figure 2a by testing the main effects of GPE dose (- or +) and LPS (- or +) and their interaction (GPE x LPS) using two-way ANOVA (JMP Version 6.03, SAS Institute, Cary, NC). A one-way ANOVA was conducted for data in Figures 2b, 4b, and 5. Student's t tests were used to compute individual pairwise comparisons of least square means, $P < 0.05$. Data are expressed as means \pm S.E.M.

Results

Polyphenol composition of GPE

Analysis of our GPE by reverse phase HPLC by Dr. Wei Jia at the UNCG Center for Research Excellence in Bioactive Food Components, Kannapolis, NC revealed a high concentration of the polyphenol quercetin-3-glucoside (Q3G) (**Fig. 2.1**). Other polyphenols included catechins (7.3%), epicatechins (1.9%), gallic acid (1.4%), rutin (1.2%), and resveratrol (0.53%).

GPE decreases LPS-induced inflammatory gene expression

To determine the extent to which GPE attenuated markers of inflammation, differentiated U937 cells were pretreated for 1 h with GPE (0, 10, 30, or 100 $\mu\text{g mL}^{-1}$)

and stimulated with 100 ngmL⁻¹ LPS for 3 h. LPS treatment markedly increased the expression of IL-6, IL-8, IL-1 β , TNF α , and GPE decreased the induction of these genes in a dose-dependent manner (**Fig. 2.2a**). LPS-induced IP-10 was blocked by 10 μ gmL⁻¹ GPE, and COX-2 induction was attenuated to the same extent with 10 and 30 μ gmL⁻¹ GPE, and blocked by 100 μ gmL⁻¹ GPE (**Fig. 2.2a**). In the absence of LPS, GPE alone did not affect inflammatory gene expression (**Fig. 2.2a**). Because LPS-mediated induction of IP-10 and IL-6 was decreased to the greatest extent by GPE, we measured their secretion in the CM. IL-6 secretion was robustly increased by LPS, whereas IP-10 was only slightly increased (**Fig. 2.2b**). LPS-mediated IL-6 secretion was modestly decreased by 30 μ gmL⁻¹ GPE, whereas IP-10 secretion was blocked by GPE. These differences may be due to the level of secretion induced by LPS. No visual cytotoxic effects of GPE were observed (e.g., no floating cells or changes in cell morphology).

GPE decreases LPS-mediated activation of MAPK

Given the important role of MAPK in activating transcription factors that induce inflammatory gene expression, we examined the effects of GPE on the phosphorylation of MAPK in M Φ s. Differentiated U937 cells were pretreated for 1 h with GPE (0, 30, 60, or 100 μ gmL⁻¹) and stimulated with 100 ngmL⁻¹ LPS for 30 min. GPE attenuated LPS phosphorylation of JNK and p38 in a dose dependent manner (**Fig. 2.3**). In the absence of LPS, GPE alone did not affect phosphorylation of JNK or p38. Phosphorylation levels of ERK were also measured, but GPE did not attenuate LPS phosphorylation of ERK (data not shown).

GPE decreases LPS-mediated NFκB, AP-1, and Elk-1 activation

To determine the extent to which GPE prevented LPS activation of the inflammatory transcription factors NFκB, AP-1, and Elk-1, IκBα degradation, c-Jun, and Elk-1 phosphorylation and NFκB reporter activity were determined in differentiated U937 cells. Pretreatment of MΦs with 100 μgmL⁻¹ GPE attenuated LPS-mediated IκBα degradation (**Fig. 2.4a**). GPE decreased LPS-stimulated phosphorylation of c-Jun in a dose dependent manner (**Fig. 2.4a**). Pretreatment of MΦs with 60 μgmL⁻¹ and 100 μgmL⁻¹ GPE decreased LPS-stimulated Elk-1 phosphorylation. In the absence of LPS, GPE had no effect on IκBα degradation, but slightly increased phosphorylation of c-Jun and Elk-1 (**Fig. 2.4a**). Finally, 10 μgmL⁻¹ GPE and 0.1 μM Rosiglitazone (i.e., BRL, a positive control) blocked NFκB reporter activation by LPS (**Fig. 2.4b**).

GPE pretreatment of MΦs decreases MΦ-CM-mediated inflammation and insulin resistance in human adipocytes

Several studies have reported cross-talk between murine MΦs and adipocytes; i.e., activated MΦs can inflame adipocytes and vice-versa (Suganami et al. 2005). Therefore, we hypothesized that GPE pretreatment of LPS-treated MΦs would prevent MΦ-CM-mediated inflammation and insulin resistance in human adipocytes. Consistent with our hypothesis, 30 μgmL⁻¹ GPE pretreatment decreased MΦ-mediated 1) induction of inflammatory gene expression (**Fig. 2.5a**), 2) activation of an NFκB reporter (**Fig.**

2.5b), and 3) impairment of insulin-stimulated glucose uptake (**Fig. 2.5c**) in human adipocytes.

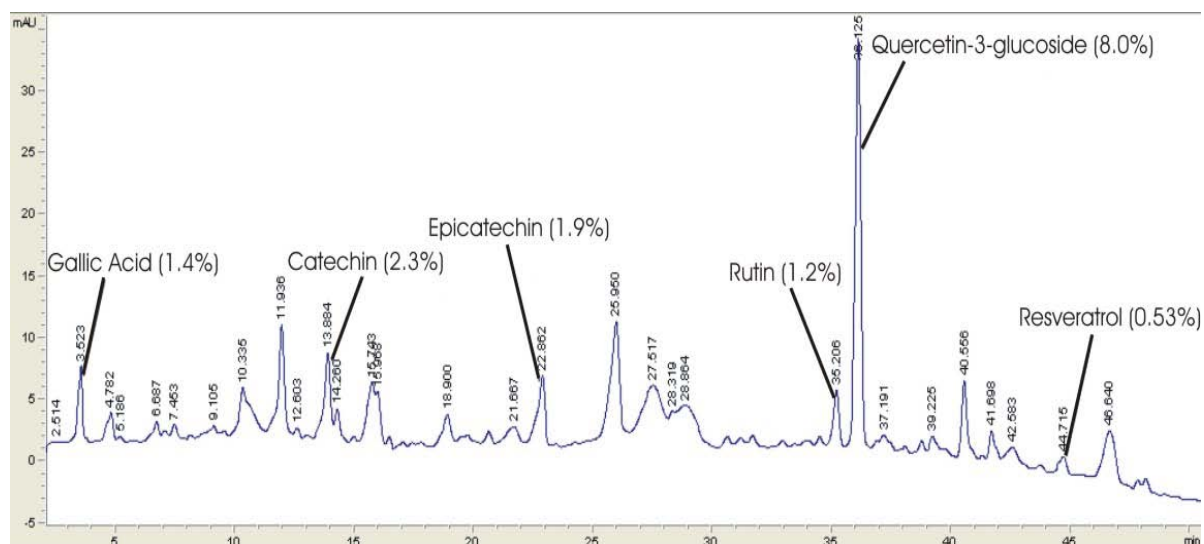


Figure 2.1 Polyphenol composition of GPE. Reverse phase HPLC of GPE indicates quercetin-3-glucoside is a major component of GPE (conducted by Dr. Jia).

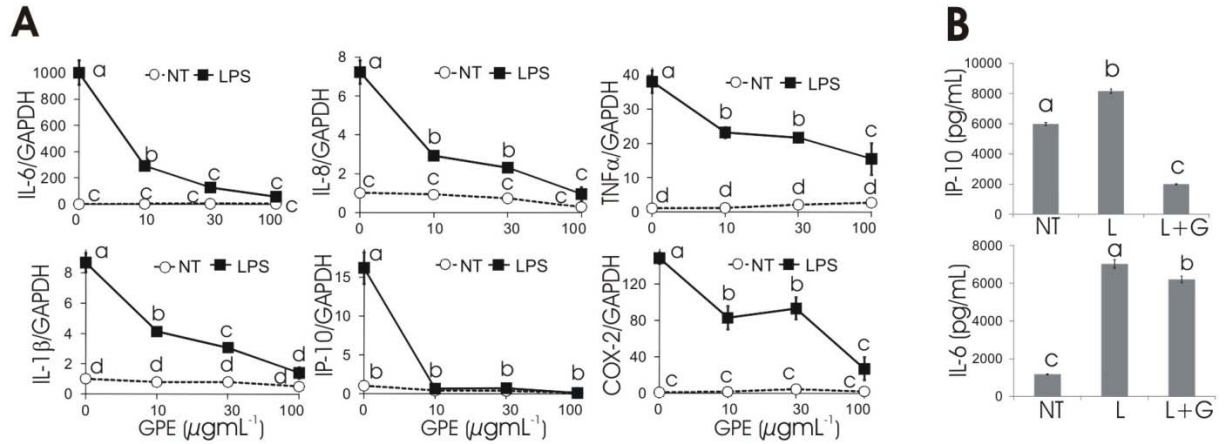


Figure 2.2 GPE blocks LPS-mediated inflammatory gene expression and protein secretion in human MΦs. (a) Differentiated U937 cultures were pretreated with 0, 10, 30, or 100 μg/mL⁻¹ GPE for 1 h and then treated with 100 ng/mL⁻¹ LPS for 3 h. The mRNA levels were measured by qPCR. Data are representative of three independent experiments. Means (± SE; n=3) without a common letter differ (P<0.05). (b) Cultures were pretreated ± 30 μg/mL⁻¹ GPE (G) for 1 h and then treated ± 100 ng/mL⁻¹ LPS (L) for 12 h. Levels of IP-10 and IL-6 in CM were measured using BioRad's Multi-Plex System. Means (± SE; n=6) without a common letter differ, P<0.05. Data are representative of two independent experiments.

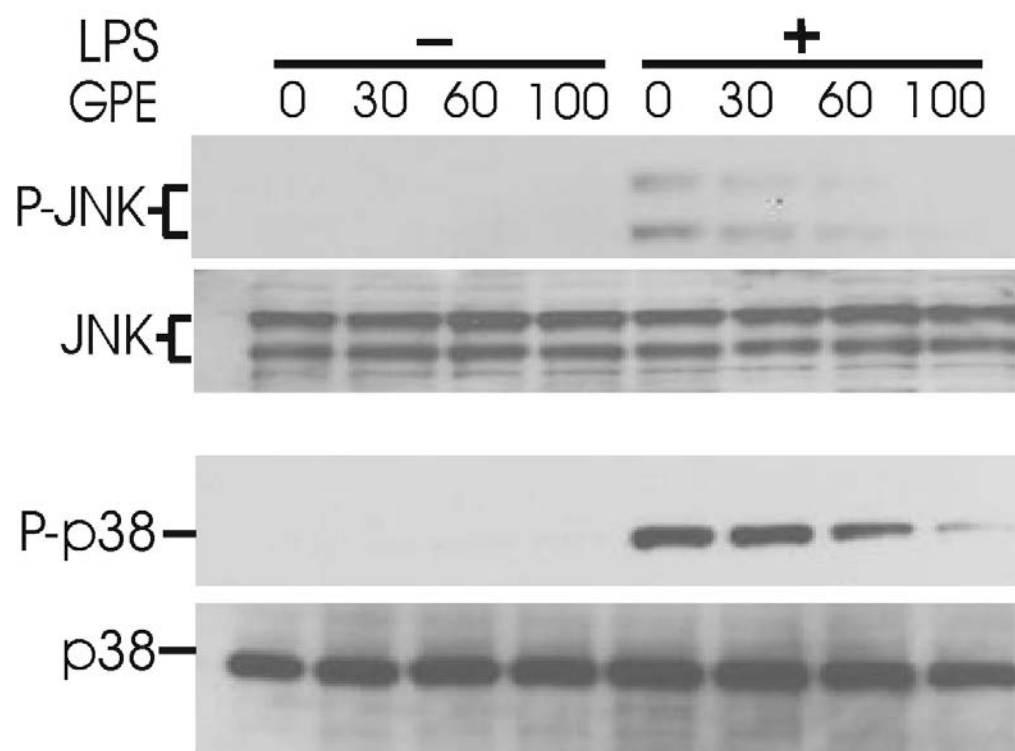


Figure 2.3 GPE attenuates LPS-mediated MAPK activation in MΦs. Differentiated U937 cells were pretreated 0, 30, 60, or 100 $\mu\text{g mL}^{-1}$ GPE for 1 h and then treated with 100 ng mL^{-1} LPS for 30 min. Proteins were immuno-blotted and probed with phospho-(P) or non-P specific antibodies for JNK and p38. Data are representative at least three independent experiments.

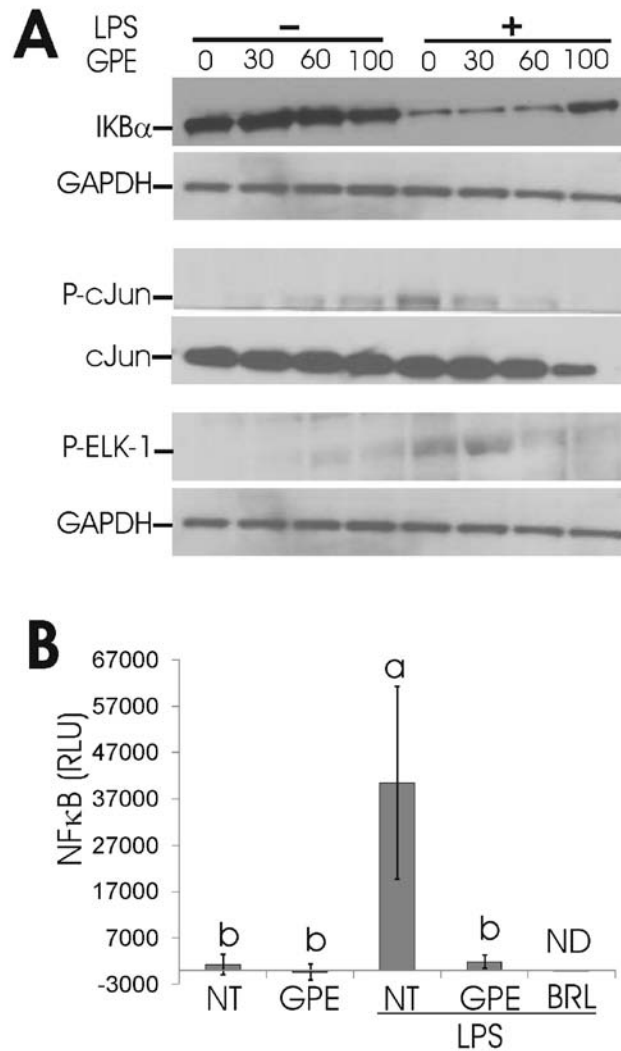


Figure 2.4 GPE attenuates LPS-mediated NFκB, c-Jun, and Elk-1 activation in MΦs. (a) Differentiated U937 cells were pretreated 0, 30, 60, or 100 $\mu\text{g mL}^{-1}$ GPE for 1 h and then treated with 100 ng mL^{-1} LPS for 30 min. Proteins were immuno-blotted and probed with phospho-(P) or non-P specific antibodies for, IκBα, c-Jun, Elk-1, or GAPDH. Data represent at least two independent experiments. (b) Cultures were pretreated with 10 $\mu\text{g mL}^{-1}$ GPE or 0.1 μM BRL (Rosiglitazone) for 1 h and then treated with 10 ng mL^{-1} LPS for 8 h. NFκB reporter activity was measured using a luciferase assay. NFκB levels for the LPS+BRL treatment were non-detectable (ND). Means (\pm SE; n=5-6) without a common letter differ, $P < 0.05$. Data represent three independent experiments.

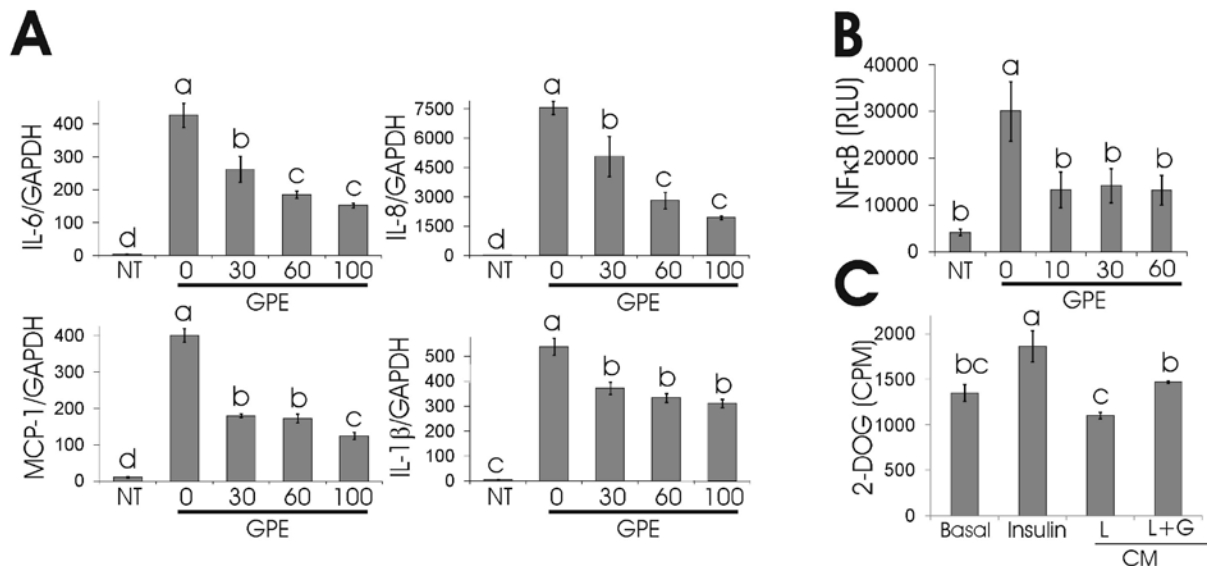


Figure 2.5 GPE decreases the inflammatory and insulin desensitizing capacity of MΦ-CM in human adipocytes. Differentiated U937 cells were pretreated with 0, 10, 30, 60, or 100 $\mu\text{g mL}^{-1}$ GPE for 1 h and then treated with 100 ng mL^{-1} LPS for 3 h. MΦ-CM was collected and used to treat adipocytes for 3-24 h at a 1:1 ratio in AM-1. (a) Adipocytes were treated with MΦ-CM for 3 h and then harvested for qPCR analysis ($n=3$). (b) Adipocytes transfected with an NFκB reporter were treated with MΦ-CM for 24 h and then were assayed for luciferase activity ($n=4-6$). (c) Adipocytes were treated with LPS-challenged MΦ-CM (L) or LPS-challenged MΦ-CM pretreated with GPE (L+G) for 24 h and 2-DOG uptake was measured ($n=3-6$). Means (\pm S.E.M.) without a common letter differ, $P<0.05$. Data in (a)-(c) are representative of at least two independent experiments.

Discussion

In this study, we demonstrated that GPE attenuated LPS-induced expression of IL-6, IL-8, IL-1 β , IP-10, TNF α , and COX2 and subsequent secretion of IL-6 and IP-10 in human M Φ s. GPE also attenuated LPS activation of JNK and p38, and the transcription factors NF κ B, AP-1 (i.e., c-Jun), and Elk-1. Furthermore, we showed that GPE decreased inflammatory gene expression (i.e., IL-6, IL-8, IL-1 β , and MCP-1) and NF κ B activity, and improved insulin-stimulated glucose uptake in human adipocytes treated with LPS-challenged M Φ -CM. Taken together, these findings demonstrate that GPE inhibits LPS-mediated activation of inflammatory MAPK and transcription factors that induce inflammatory gene expression and protein secretion in cultures of human M Φ s. This suppression of inflammatory protein secretion by GPE likely contributed to the decreased the ability of CM from LPS-stimulated M Φ s to cause inflammation and insulin resistance in human adipocytes. Based on these data, we propose that GPE directly attenuates LPS activation of MAPK, NF κ B, and AP-1 in M Φ s, thereby preventing the induction and secretion of inflammatory cytokines (e.g., IL-6) and chemokines (i.e., IP-10). This decreased production of inflammatory mediators prevents M Φ s from causing inflammation and insulin resistance by attenuating the activation of NF κ B in adipocytes.

Consistent with these data, increased levels of LPS have been found in the plasma of high-fat fed obese mice (Cani et al. 2009), which contributes to inflammation and insulin resistance. LPS activates MAPK, NF κ B, and AP-1, increasing the release of inflammatory cytokines and chemokines (Terra et al. 2007, Jang et al. 2008, Chen et al.

2007). In obesity, the increased production of these inflammatory mediators originates mainly from infiltrating MΦs in WAT (Xu et al. 2003, Weisberg et al. 2003). MΦ-secreted factors have been shown to increase inflammation and decrease insulin-stimulated glucose uptake in adipocytes (Lumeng et al. 2007, Permana et al. 2006). Consistent with these findings, we found that LPS increased the activation of MAPK, NFκB, and AP-1 in human MΦs, increasing their capacity to cause inflammation and insulin resistance in primary human adipocytes.

Several grape products have been shown to reduce markers of inflammation or oxidative stress associated with cardiovascular disease *in vivo*, *ex vivo*, and *in vitro* (reviewed in Perez-Jimenez et al. 2008 and Leifert et al. 2008). For example, consumption of red grape juice by hemodialysis patients, who suffer from hemodialysis-induced oxidative stress, reduced plasma levels of oxidized LDL and *ex vivo* neutrophil NAPDH oxidase activity (Castilla et al. 2008). Similarly, consumption of red grape juice decreased markers of oxidative stress in healthy adults (O'Bryne et al. 2002). Consumption of grape powder by adult women decreased the levels of plasma TNFα and urinary F2-isoprostanes, markers of inflammation and oxidative stress, respectively (Zern et al. 2005). Similarly, adults consuming red wine had lower urinary levels of PGF_{2α} levels and higher plasma levels of polyphenols compared to those consuming white wine or no wine (controls) (Pignatelli et al. 2006).

Salt-sensitive, hypertensive Dahl rats supplemented with grape powder had decreased cardiac oxidative damage and plasma levels of IL-6 and TNFα compared to unsupplemented control rats (Seymour et al. 2008). Zucker rats fed a high fat diet

supplemented with grape seed procyanidins had lower plasma levels of C-reactive protein (CRP) and lower WAT mRNA levels of CRP, TNF α , and IL-6 compared to high fat fed control rats (Terra et al. 2009). Apolipoprotein E deficient mice, who suffer from atherosclerosis and oxidative stress, supplemented with grape powder had a lower incidence of atherosclerotic plaque formation and decreased markers of oxidative stress compared to controls (Zern et al. 2005). Furthermore, consumption of grape powder by apolipoprotein E deficient mice decreased the capacity of their peritoneal M Φ s to oxidize LDL. Consistent with these data, grape seed proanthocyanidin supplementation to cholesterol-fed hamsters decreased M Φ -mediated, aortic foam cell development by 50% compared to the unsupplemented controls (Vinson et al. 2002).

In vitro, grape seed procyanidins decreased cholesterol and TG accumulation in M Φ -derived foam cells (Terra et al. 2009). In addition, human M Φ (THP-1 cell line) and adipocytes (SGBS cell line) treated with grape seed procyanidins had reduced expression of IL-1 and MCP-1 and decreased activation of NF κ B following stimulation with LPS or TNF α , respectively (Chacón et al. 2009). Consistent with these findings, our data show that GPE attenuates LPS-mediated inflammatory gene expression in M Φ s, possibly by decreasing activation of JNK, p38, c-Jun, Elk-1, or NF κ B.

Because grapes contain a wide array of phytochemicals, the specific bioactive components responsible for the anti-inflammatory properties of grapes or GPE remain to be elucidated. Two promising candidates in grapes are resveratrol and quercetin which, based on our reverse phase HPLC analysis, were found to be present in our GPE. For example, resveratrol attenuated the phagocytosis of bacteria by M Φ s via decreased NF κ B

activation (Iyori et al. 2009) and suppressed monocyte adhesion to human umbilical vein endothelial cells (Moon et al. 2006). Furthermore, quercetin reduced the levels of inflammatory markers in LPS treated MΦ U937 cells (Okoko et al. 2009) and suppressed the degradation of IκBα and the phosphorylation of p38 and Akt in LPS-stimulated bone marrow-derived MΦs (Kaneko et al. 2008).

One potential mechanism by which GPE may block inflammation is by increasing peroxisome proliferator-activated receptor (PPAR)-γ expression or activation. PPAR-γ has been described as a general negative regulator of MΦ activation and has been implicated in muscle and hepatic insulin sensitivity and in inflammation. PPAR-γ agonists such as TZDs reduce inflammatory gene expression in MΦ (Necela et al. 2008) and when administered before the onset of inflammation, TZDs exhibit beneficial effects on experimental models of inflammation such as colitis (Su et al. 1999, Desreumaux et al. 2001, Saubermann et al. 2002, Katayama et al. 2003, Lytle et al. 2005, Schaefer et al. 2005), atherosclerosis (Nagy et al. 1998, Tontonoz et al. 1998, Li et al. 2000, Zhang et al. 2004), asthma (Hammad et al. 2004, Woerly et al. 2003, Trifillieff et al. 2003), psoriasis (Demerjiann et al. 2006), myocarditis (Hasegawa et al. 2005, Yuan et al. 2004) and allergic encephalomyelitis (Natarajan et al. 2002, Diab et al. 2002). In addition, anthocyanins such as Cyanidin-3-*O*-β-glucoside have also been shown to enhance the expression and transcriptional activities of PPAR-γ in MΦ (Wang et al. 2008, Xia et al. 2005). The underlying mechanisms are not fully elucidated but it has been suggested that PPAR-γ activation exerts its anti-inflammatory function by trans-repressing the NFκB and MAPK pathways (Szanto et al. 2008, Varga et al. 2008). Thus, GPE may be

attenuating LPS-mediated activation of NFκB and MAPKs by acting as a PPAR-γ agonist. Studies are underway in our laboratory to determine the extent to which the phytochemicals resveratrol and quercetin contribute to the anti-inflammatory and insulin-sensitizing properties of GPE. Other polyphenols in GPE that may also decrease inflammation include anthocyanins and flavanols (Seymour et al. 2008).

Collectively, these data demonstrate that GPE attenuates LPS-mediated inflammatory gene expression and protein secretion in human MΦs, possibly by suppressing the activation of JNK, p38, c-Jun, Elk-1, or NFκB. Furthermore, GPE decreased the inflammatory capacity of LPS-challenged MΦ-CM, attenuating its ability to cause inflammation and insulin resistance in primary human adipocytes. Future *in vivo* studies are needed to recapitulate these *in vitro* findings, and determine the bioactive component(s) in GPE.

CHAPTER III

EPILOGUE

Obesity produces a state of chronic, low-grade inflammation that contributes to the pathogenesis of numerous diseases including type 2 diabetes, atherosclerosis, and hypertension. Although the exact cellular origin of this inflammatory response remains to be fully elucidated, recent attention has focused on the role infiltrating MΦs play in WAT inflammation. Preventing this inflammatory response in MΦs with polyphenol-rich foods like grapes could help prevent or delay the development of obesity-related diseases. Indeed, consumption of polyphenol-rich foods like grapes or their by-products has been reported to prevent inflammatory-related diseases. Therefore, this project examined the extent to which GPE attenuated inflammatory markers in human MΦs and the underlying mechanisms behind this effect.

Based on my findings, I propose that GPE attenuates LPS activation of MAPK, NFκB, and AP-1 in MΦs, thereby preventing the induction and secretion of inflammatory mediators. This decreases the ability of MΦs to cause inflammation and insulin resistance in adipocytes. These results have led to the development of the following new questions 1) What are the bioactive components in GPE contributing to anti-inflammatory effects? 2) What effects do GPE or its bioactive components have on PPARγ in human MΦs, and 3) Can these results be recapitulated *in vivo*?

Q1. What are the bioactive components in GPE contributing to its anti-inflammatory effects?

Two polyphenols that have been shown to possess anti-inflammatory properties and are present in GPE include QUE (i.e., quercetin-3-glucoside (Q3G)) and RSV. Because QUE was found to be more abundant than RSV, I investigated the ability of QUE to attenuate LPS-mediated inflammatory gene expression in human MΦs. I used QUE instead of Q3G because they both equally decreased IL-6 and IP-10 gene expression in LPS-treated MΦs. Thus, I repeated the studies conducted in Chapter II with QUE instead of GPE. Consistent with GPE, QUE decreased LPS induction of IL-6 and IP-10 gene expression and protein secretion into CM (**Fig. 3.1a and 3.1b**). However, QUE did not attenuate LPS-induced IL-1 β , IL-8, COX-2, or TNF α gene expression (data not shown). Furthermore, QUE did not decrease LPS activation of MAPK, NF κ B, or AP-1 in MΦs, nor did it decrease the capacity of LPS-challenged MΦ-CM from causing inflammation and insulin resistance in human adipocytes (data not shown). Interestingly, in the absence of LPS, QUE attenuated IL-6, IP-10, TNF α , IL-8, IL-1 β , and COX-2 gene expression (**Fig. 3.2**). QUE also prevented I κ B α degradation and decreased JNK and cJun phosphorylation in the absence of LPS (**Fig. 3.3**). In addition, QUE pretreatment prevented MΦ-mediated insulin resistance in human adipocytes (**Fig. 3.4**). Studies are underway to determine if QUE pretreatment prevents inflammatory gene expression in adipocytes challenged with MΦ-CM.

Surprisingly, my recent preliminary data (n=2) shows that QUE and Q3G were not detectable in human MΦs exposed to GPE for 3-24 h. However, ng levels QUE (0.1 and

0.2 ng) and Q3G (0.03 and 0.01 ng) were detectable in MΦs exposed to 30 uM QUE or 60 uM Q3G for 8 or 24 h, respectively. These analyses were performed by Dr. Jia using HPLC/MS. The extent to which QUE or Q3G in GPE may have been metabolized into other metabolites is unknown, as we did not measure the disappearance of QUE or Q3G from the media of cells over time. Notably, 3.5 ng, 3.8 ng, and 4.6 ng of trans-RSV were detected in cells exposed to GPE for 3, 8, and 24 h, respectively. However, these data do not mean that RSV is a more bioactive component than QUE from GPE, because the QUE could be rapidly interacting with cellular targets, thereby being transformed into other types of QUE metabolites or species. These studies need to be repeated to confirm these results.

Q2. What effects do GPE or its bioactive components have on PPAR γ in human MΦs?

One alternate mechanism by which GPE or its bioactive compounds decrease inflammation is by increasing PPAR γ activity, which has been reported to antagonize NF κ B and AP-1 (Ricotte et al 2007, Straus et al 2007, Ghisletti et al 2007). Based on my preliminary data showing LPS decreased PPAR γ mRNA in MΦs, I would expect LPS to decrease PPAR γ protein levels and activity as well. Thus, I will examine the effects of GPE and its bioactive compounds on PPAR γ expression and activity in LPS-treated MΦs. I have conducted transient transfections studies in MΦs with a PPRE reporter construct; however I had problems getting the reporter transfected into the MΦs. Therefore, an alternate approach would be to use an electrophoretic mobility shift assay

(EMSA) to examine the extent to which LPS decreases PPAR γ activity and whether GPE attenuates this effect.

Q3. Can these results be recapitulated in vivo?

Studies are underway in our research group to determine the extent to which grape powder (GP) polyphenols are absorbed in normal mice and prevent inflammation and insulin resistance in high fat (HF)-fed obese mice. To examine the absorption of GP polyphenols, mice will be gavaged with GP and their blood will then be analyzed for GP polyphenols and their metabolites using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrophotometry (UPLC-QTOFMS). The effects of GP polyphenols on inflammation and insulin resistance in HF-fed obese mice will be examined by feeding obese mice a HF diet containing GP for 16 wks. Blood will be taken at different times during this period to assess fasting glucose and insulin levels. At the end of this study, mice will be euthanized and serum levels of inflammatory markers (IL-6, IL-8, MCP-1, IL-1 β , TNF α , free fatty acids) and proteins associated with insulin resistance (i.e, decreased adiponectin) will be measured. In addition, epididymal fat pads will be analyzed for mRNA markers of M Φ recruitment, inflammation, and insulin resistance. From these studies, I hypothesize that several polyphenols detected in our GP extract (e.g, QUE, RSV) will indeed be detectable in the blood of mice. Furthermore, I hypothesize that GP will reduce inflammatory gene expression and M Φ recruitment into WAT and improve insulin sensitivity in WAT.

In conclusion, the experiments discussed in this section would help to identify which polyphenols in GPE are absorbed and metabolized. Studies will then be conducted to determine if these candidate polyphenols possess anti-inflammatory properties and if so, the mechanisms behind these effects can be elucidated through further *in vitro* studies. Most likely, we will find a number of polyphenols in GPE that have additive or synergistic effects in decreasing chronic, low grade inflammation.

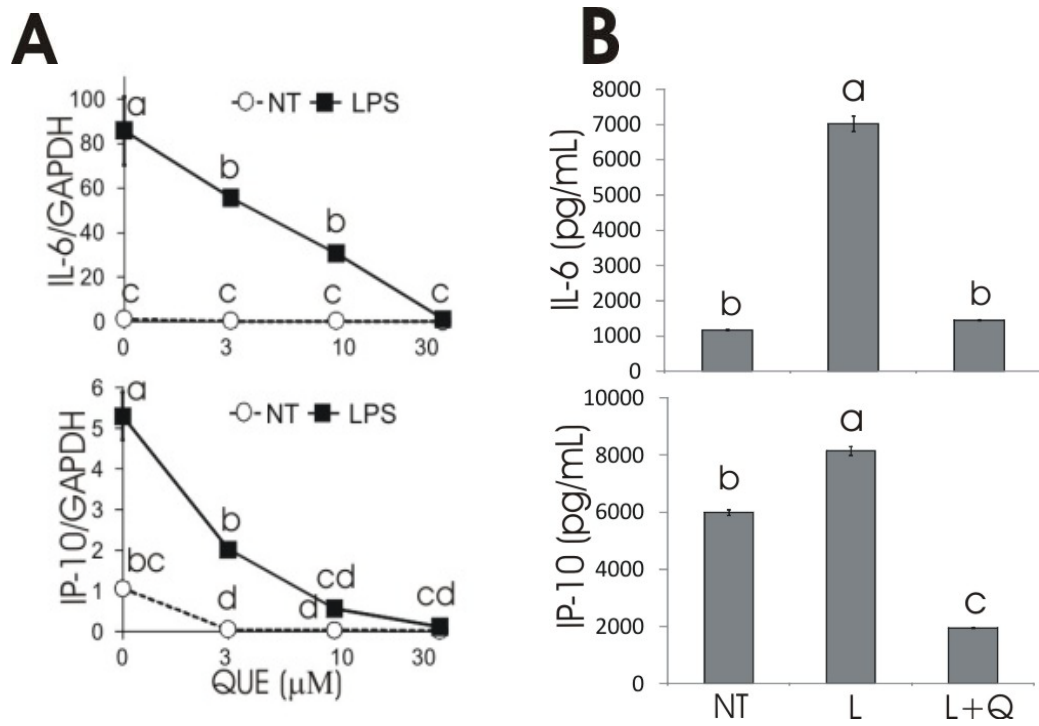


Figure 3.1 QUE blocks LPS-mediated IP-10 and IL-6 gene expression and protein secretion in human MΦs. (a) Differentiated U937 cultures were pretreated with 0, 3, 10, or 30 μM QUE for 1 h and then treated with 100 ngmL⁻¹ LPS for 3 h. The mRNA levels were measured by qPCR. Data are representative of three independent experiments. Means (± SE; n=3) without a common letter differ (P<0.05). (b) Cultures were pretreated ± 30 μM QUE (Q) for 1 h and then treated ± 100 ngmL⁻¹ LPS (L) for 12 h. Levels of IP-10 and IL-6 in CM were measured using BioRad's Multi-Plex System. Means (± SE; n=6) without a common letter differ, P<0.05. Data are representative of two independent experiments.

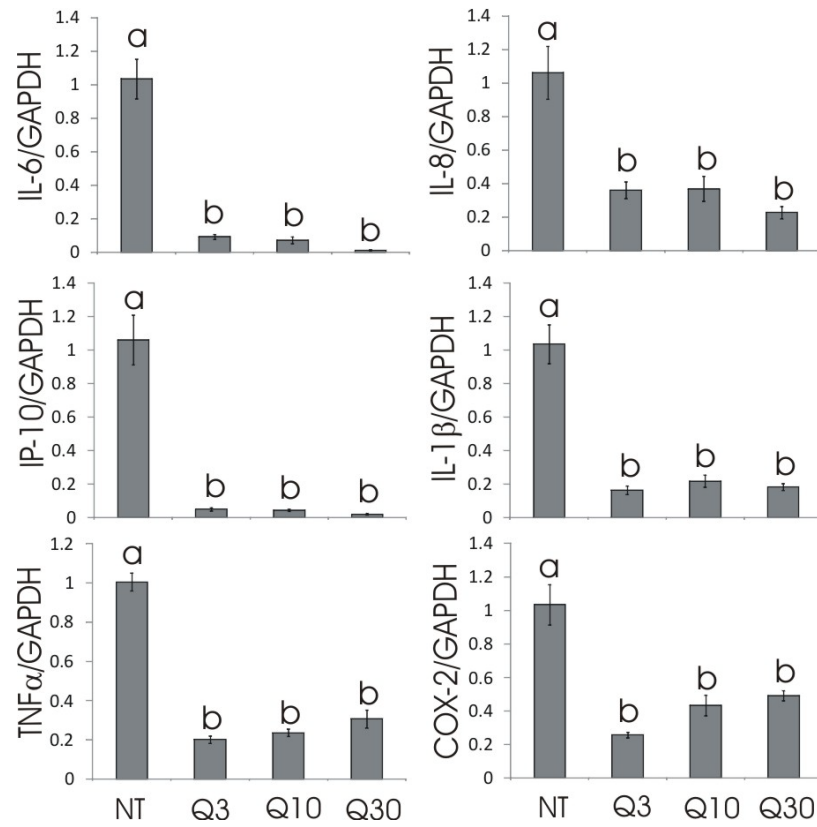


Figure 3.2 QUE attenuates inflammatory gene expression in human MΦs. Differentiated U937 cultures were treated with 0, 3, 10, or 30 μ M QUE (Q) for 4 h. The mRNA levels were measured by qPCR. Data are representative of three independent experiments. Means (\pm SE; n=3) without a common letter differ ($P<0.05$)

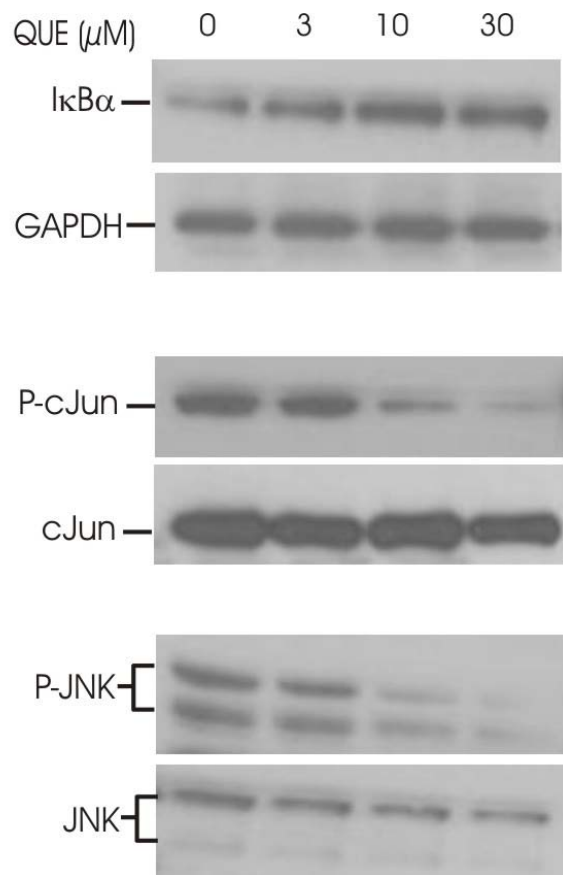


Figure 3.3 QUE attenuates I κ B α degradation and JNK and cJun phosphorylation. Differentiated U937 cells were treated with 0, 3, 10, or 30 μ M QUE. Proteins were immuno-blotted and probed with phospho-(P) or non-P specific antibodies for, I κ B α , c-Jun, JNK, or GAPDH. Data are representative of two independent experiments.

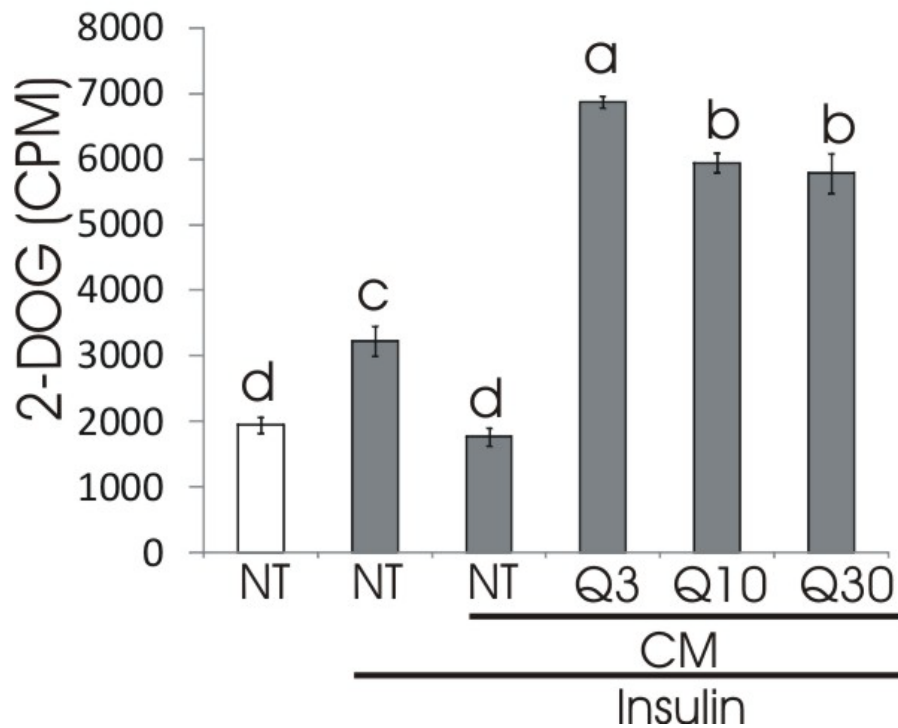


Figure 3.4 QUE decreases the insulin desensitizing capacity of MΦ-CM in human adipocytes. Differentiated U937 cells were treated with 0 (NT), 3, 10, 30 μ M QUE for 5 h and MΦ-CM was collected and used to treat adipocytes for 24 h at a 1:1 ratio in AM-1. 2-DOG uptake was measured (n=4). Means (\pm SE; n=4) without a common letter differ, $P < 0.05$. Data are representative of one experiment.

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